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TULA proteins bind to ABCE-1, a host factor of HIV-1 assembly, and inhibit HIV-1 biogenesis in a UBA-dependent fashion

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Abstract

TULA, a recently identified UBA- and SH3-containing protein, has previously been shown to regulate cell signaling through protein tyrosine kinases. In order to search for novel functions of TULA, we identified, using mass spectrometry, proteins associated with TULA. ABCE-1 also known as RLI and HP68, a host factor of HIV-1 assembly, was found among TULA-associated proteins in these experiments. Considering an important role of ABCE-1 in HIV-1 assembly, we were compelled to analyze the effect of TULA on HIV-1 biogenesis. Our study provides evidence that TULA proteins substantially inhibit production of both sub-genomic and full-length HIV-1 viral particles and that the effect of TULA is dependent on UBA domain-mediated interactions. The primary role of ABCE-1 in the effect of TULA appears to be the recruitment of TULA to the sites of HIV-1 assembly where TULA interferes with the late steps of the HIV-1 life cycle, most likely by disrupting essential ubiquitylation-dependent events that remain to be identified.
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Keywords: HIV-1; TULA; ABCE-1; Ubiquitin; Gag; UBA; Mass spectrometry

Introduction

We recently identified TULA among multiple proteins co-purifying with c-Cbl from a T-cell line (Feshchenko et al., 2004). TULA contains several distinct domains—a UBA domain capable of binding to ubiquitin and ubiquitylated proteins, an SH3 domain capable of binding to proline-rich sequences, including those of Cbl proteins, and a region initially termed HCD, which has homology to phosphoglyceromutases (PGM) (Carpino et al., 2004; Feshchenko et al., 2004; Kowanetz et al., 2004; Wattenhofer et al., 2001) (Fig. 1). A mouse ortholog of TULA (Sts-2) was identified, and mice deficient for this protein and the second protein of this family were generated (Carpino et al., 2002, 2004). Analysis of TULA expression in tissues and cell lines demonstrated that this protein is expressed primarily in T and B lymphocytes (Carpino et al., 2004; Feshchenko et al., 2004). The second protein of this family Sts-1 (TULA-2) is expressed ubiquitously (Carpino et al., 2002, 2004). (In this report, we will use the term TULA for consistency with our previous studies; Feshchenko et al., 2004.)

Several reports have implicated TULA in the regulation of cell signaling mediated by protein tyrosine kinases (PTKs), in which the UBA domain of TULA proteins appears to play an

Abbreviations: ABCE-1, ATP-binding cassette protein family E member 1; FBS, fetal bovine serum; GFP, green fluorescent protein; HP68, human protein 68 kDa; LC-MS, liquid chromatography-mass spectrometry; ODG, β-octyl-D-glucoside; PBS, phosphate-buffered saline; PGM, phosphoglyceromutase; PTK, protein tyrosine kinase; RLI, RNase L inhibitor; SH3, Src homology 3 domain; Sts, suppressor of T-cell receptor signaling; TULA, T-cell ubiquitin ligand; UBA, ubiquitin-associated domain.

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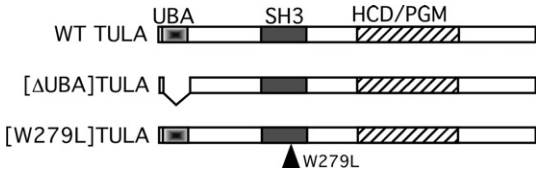

Fig. 1. Schematic view of TULA and its mutants. UBA is a ubiquitin-associated domain. SH3, an Src homology domain 3, binds to proline-rich motifs. HCD/PGM is a region homologous to phosphoglyceromutases. [ΔUBA]TULA lacks UBA as a result of a deletion. The W279L mutation inactivates TULA's SH3 domain.

essential role (Carpino et al., 2004; Feshchenko et al., 2004; Kowanetz et al., 2004). Since TULA proteins have been described only recently, it is possible that they exert biologically important effects other than the regulation of PTK-mediated signaling. In order to address the issue of possible novel functions of TULA, we decided to identify proteins associated with TULA and to assess the involvement of TULA in cellular events mediated by them. One of the proteins identified in these experiments is the cellular factor of HIV-1 assembly termed RLI, HP68 and ABCE-1 by different groups (Bisbal et al., 1995; Kerr, 2004; Zimmerman et al., 2002).

Although HIV-1 Gag has self-assembling properties, multiple studies indicate that host factors are involved in HIV-1 assembly in the cell (Gottlinger, 2001; Morikawa, 2003). Furthermore, studies in a cell-free system suggested that the assembly of HIV-1 particles is a stepwise, energy-dependent process (Lingappa et al., 1997). The search for ATP-binding molecular chaperones interacting with HIV-1 Gag led to the discovery of HP68 (Human Protein 68 kDa) (Zimmerman et al., 2002). HIV-1 Gag binds to HP68 immediately after translation and remains associated with it in immature HIV-1 assembly intermediates until the beginning of HIV-1 Gag processing (Dooher et al., 2007; Zimmerman et al., 2002). Consistent with these findings, assembly-incompetent HIV-1 Gag fails to bind to HP68, whereas Gag from processing-defective HIV-1 exhibits prolonged association with HP68 (Dooher et al., 2007; Lingappa et al., 2006; Zimmerman et al., 2002). Wild-type, but not assembly-incompetent HIV-1 Gag exhibits substantial co-localization with HP68 at the HIV-1 assembly sites on the plasma membrane (Dooher et al., 2007; Zimmerman et al., 2002). Finally, a truncated form of HP68 blocks virion production in vivo, and HP68 depletion significantly hinders HIV-1 Gag assembly in vitro (Zimmerman et al., 2002).

HP68 contains two ATP/GTP-binding domains and may act by bringing ATP to the immature HIV-1 intermediates (Zimmerman et al., 2002) or by inducing conformational changes in HIV-1 Gag, which are required for the late steps of capsid formation (Datta et al., 2007), since the crystal structure of HP68 suggests that these domains undergo an ATP-driven clamp-like motion around a hinge region as if in a mechano-chemical enzyme (Karcher et al., 2005).

HP68 was initially identified as the RNase L inhibitor (RLI) (Bisbal et al., 1995). RNase L is an effector enzyme of the cellular system that utilizes 2′,5′-linked oligoadenylates as a major regulator, which is involved in the interferon-induced antiviral and antiproliferative response (Bisbal et al., 2001; Bisbal and Silverman, 2007; Flodstrom-Tullberg et al., 2005; Silverman, 1994). Upregulation of the RLI expression by viruses, including HIV-1 (Martinand et al., 1999), suggests that HP68/RLI may have a two-fold positive effect on HIV-1 replication; first, as a host factor essential for capsid assembly, and second, as a negative regulator of the antiviral enzyme RNase L. Considering that bioinformatics analysis argues that the most appropriate name for HP68/RLI is ABCE-1, since this protein is the sole member of the ATP-binding cassette (ABC) protein family E (Kerr, 2004), we will use the term ABCE-1 throughout the text.

Results

Binding of TULA to ABCE-1

To date, very few TULA-binding partners are known (Bertelsen et al., 2007; Feshchenko et al., 2004; Kowanetz et al., 2004). In order to identify novel TULA-associated proteins, FLAG-tagged TULA was overexpressed in 293T cells, which do not express endogenous TULA (Feshchenko et al., 2004), and immunoprecipitated using anti-FLAG antibodies. The protein content of the obtained immune complex was analyzed using liquid chromatography-tandem mass spectrometry (LC-MS/MS). In each of three replicate experiments we identified ABCE-1 (Table 1), which has previously been shown to be a key host factor of HIV-1 assembly (Dooher and Lingappa, 2004; Lingappa et al., 2006; Zimmerman et al., 2002). Considering the importance of cellular factors involved in HIV-1 biogenesis, we decided to further analyze a possible functional link between TULA and ABCE-1.

To verify association of TULA and ABCE-1 using an independent approach, we co-expressed FLAG-TULA and Myc-ABCE-1 in 293T cells and immunoprecipitated TULA with anti-FLAG antibody. This resulted in robust co-immunoprecipitation of ABCE-1 with TULA (Fig. 2A). When only TULA was overexpressed, endogenous ABCE-1, which is expressed ubiquitously (Aubry et al., 1996), also co-immunoprecipitated with TULA in a dose-dependent fashion, mirroring the level of TULA in the cells (Fig. 2B). To analyze the role of UBA and SH3, two interactive domains of TULA, in this interaction, we compared wild-type TULA and its mutant forms lacking either UBA ([ΔUBA]TULA) or functional SH3 ([W279L]TULA)

Table 1
ABCE1-specific peptides identified in TULA immune complexes

Peptide sequence	Amino acids	No. of experiments
ILEDDLK	158–165	1/3
GTVGSILDR	182–190	1/3
NVEDLSGGELQR	213–224	2/3
VIVFDGVPSK	532–541	3/3

Immunoprecipitation with subsequent identification of associated proteins was carried out as described in Materials and methods. Three individual experiments were performed. The number of experiments in which a particular peptide was detected is shown in the third column. Amino acid residues are numbered according to the NCBI gi#108773782 sequence.

with regard to their ability to bind to ABCE-1. It appears that ABCE-1 co-immunoprecipitated not only with wild-type TULA, but with both mutant forms as well (Fig. 2B).

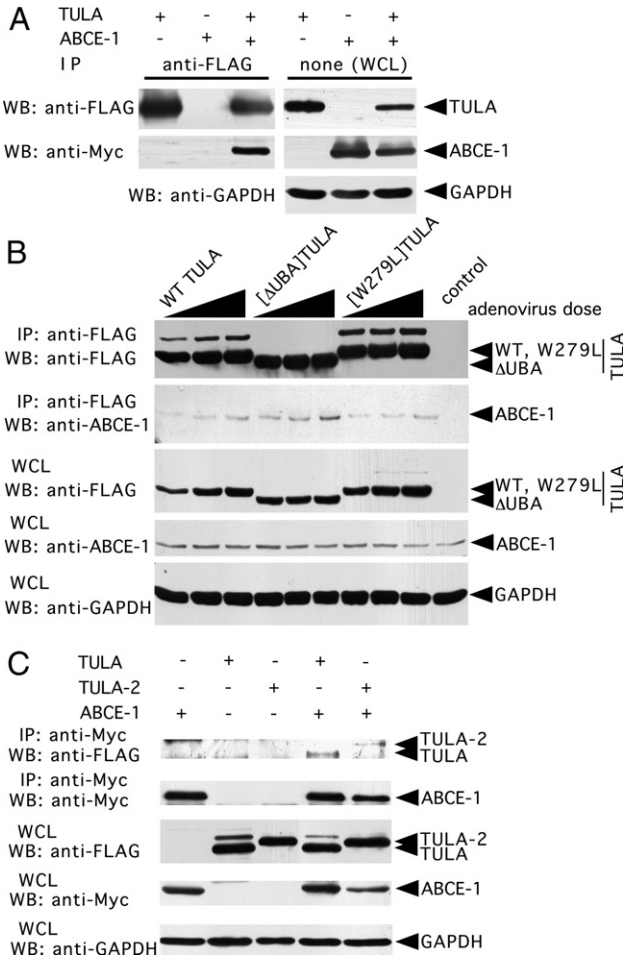

Fig. 2. Binding of TULA to ABCE-1. (A) 293T cells were transfected to overexpress FLAG-tagged wild-type TULA and/or Myc-tagged ABCE-1 as indicated at the top of the panel (8 μg of each expression plasmid per 100-mm Petri dish). A GFP expression plasmid was added to monitor the efficiency of transfection (0.5 μg per dish). Cells were homogenized in hypotonic buffer, and the obtained homogenates were solubilized with ODG/TNE buffer 48 h after transfection. Cell lysates were immunoprecipitated (IP) with anti-FLAG where indicated at the top of the panel or used without immunoprecipitation (WCL=whole cell lysates). Antibodies used in Western blotting (WB) and the proteins detected are indicated at the left and the right of the panel, respectively. The results of a representative experiment of three independent experiments are shown. (B) 293T cells were infected with recombinant adenovirus expressing wild-type (WT) or mutant TULA. Cells were lysed in TNE buffer, and their WCLs or IPs were analyzed using Western blotting as shown. The antibodies used and the proteins detected are indicated as described in panel A. In this and subsequent experiments, a minor band migrating above WT TULA and [W279L]TULA is a monoubiquitylated form of this protein (Hoeller et al., 2006). The results of a representative experiment of three independent experiments are shown. (C) 293T cells were transfected to overexpress FLAG-tagged wild-type TULA or TULA-2 with or without Myc-tagged ABCE-1 as indicated at the top of the panel (2 μg of each expression plasmid plus 0.2 μg of GFP expression plasmid per well of a 6-well plate). Cells were lysed in TNE buffer 36 h after transfection. Cell lysates were analyzed as described in panel A. The results of a representative experiment of two independent experiments are shown.

Considering that TULA belongs to a two-protein family, we assessed, using co-immunoprecipitation, the ability of its homologue (Sts-1/TULA-2) to bind to ABCE-1. The results of these experiments indicated that both TULA proteins are capable of binding to ABCE-1 (Fig. 2C). Overall, our studies indicate that TULA proteins and ABCE-1 physically interact and that neither UBA nor SH3 of TULA appears to be essential for this interaction.

Modulation of the expression of TULA proteins exerts an effect on HIV-1 production

Because ABCE-1 is a crucial host factor of HIV-1 assembly, we were compelled to study the effect of TULA proteins on HIV-1 biogenesis. First, we assessed the effect of TULA on the production of replication-incompetent GFP-expressing pseudotyped HIV-1 in 293T cells using a single-round infectivity assay in Jurkat cells. The results of these experiments showed that overexpression of wild-type TULA reduces the viral titer in supernatants ~3-fold as compared to that in the supernatants of vector control cells. Whereas [ΔUBA]TULA failed to significantly modify virus production, [W279L]TULA reduced viral titers to the same degree as wild-type TULA did (Fig. 3A). This result indicated that the SH3 domain is dispensable for the antiviral effect of TULA, while UBA is necessary for this effect. Finally, TULA-2/Sts-1 reduced viral titers to a slightly higher extent than TULA did (Fig. 3A). To assure that TULA proteins were expressed in virus-producing 293T cells, their lysates were analyzed using immunoblotting (Fig. 3B); all forms of TULA were found to be expressed at a high level. The level of GFP was constant throughout the experiment, indicating equal transfection efficiency of different samples.

To determine whether TULA proteins affect virus production or viral infectivity, we isolated virions from supernatants of virus-producing cells and assessed virus production by detecting p24, a Gag-derived protein packaged in the HIV-1 particles, using immunoblotting. These experiments indicated that production of virions by cells overexpressing either TULA or TULA-2 was significantly reduced as compared to the production of virions by vector control cells (Fig. 3B). The effects of TULA mutants in these experiments were similar to those detected using the single-round infectivity assay: wild-type TULA and [W279L]TULA reduced the production of virions to a similar degree, whereas the ability of [ΔUBA]TULA to affect virus production was substantially weakened as compared to that of wild-type TULA and of [W279L]TULA (Fig. 3B).

A simple explanation of the effect of TULA on the production of HIV-1 would be a decrease in total expression of HIV-1 Gag in cells overexpressing TULA. To examine this possibility, we analyzed HIV-1 Gag both in viral particles and cell lysates. The experiment shown in Fig. 3C demonstrated a moderate (less than 2-fold) effect exerted by TULA on the level of cellular HIV-1 Gag (both $Pr55^{Gag}$ and total HIV-1 Gag). In contrast, the effect of TULA on the amount of p24 and p17 proteins of mature HIV-1 in viral pellets was profound (up to 10-fold). These results argued that the effect of TULA on virus production is not mediated simply by an overall decrease in HIV-1

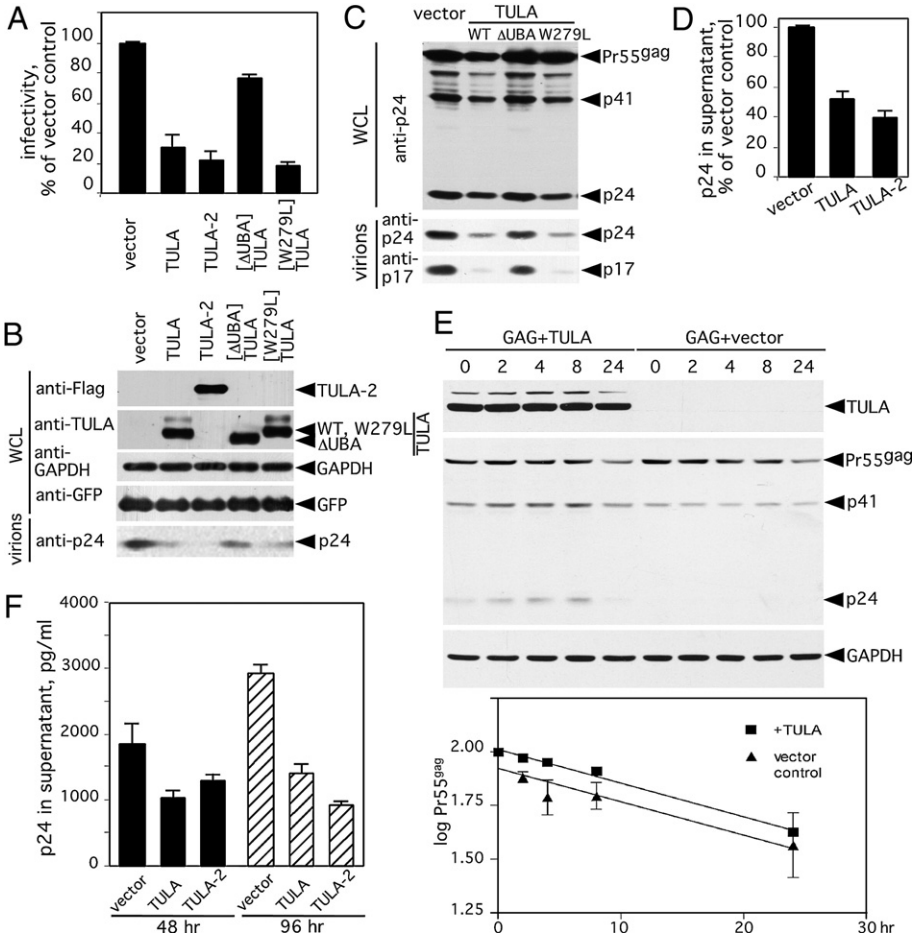

Fig. 3. Effect of TULA proteins on the production of HIV-1. (A) 293T cells were co-transfected with the three plasmids required for the production of HIV-1 VSVG-pseudotyped virus plus an expression plasmid for one of the examined proteins as indicated at the bottom of the panel (7.5 μg of each plasmid per 100-mm Petri dish). Jurkat cells (2×10^5 in a 0.5-ml sample) were transduced with 10-μl aliquots of viral supernatant (corresponding to the linear infection range) in triplicates. Percentage of infected Jurkat cells is plotted as mean ± SD; vector control = 100%. The results of a representative experiment of five independent experiments are shown. (B) TNE lysates of virus-producing 293T cells and virus pellets from the experiment shown in panel A were analyzed using Western blotting with the antibodies indicated at the left of the panel. Anti-p24 mAb AG3.0 was used to probe p24. Proteins detected are indicated at the right of the panel. (C) 293T cells were transfected with all the plasmids required for virus production and an expression plasmid for TULA, wild-type or mutant, as indicated at the top of the panel. Virions released were collected and virus-producing 293T cells were homogenized and solubilized in RIPA buffer 48 h after transfection. Both virions and cell lysates were analyzed for HIV-1 Gag expression using Western blotting. The antibodies used and the proteins detected are indicated as in panel B. Anti-p24 mAb AG3.0 was used to probe HIV-1 Gag in WCL. Goat polyclonal anti-p24 (ViroStat) was used to probe p24 in virions. The result of a representative experiment of three independent experiments is shown. (D) Cells were transfected, and their supernatants were harvested as described in panel A. Virus production was measured using a p24 antigen capture assay. The results of a representative experiment of two independent experiments are plotted as mean ± SD of duplicate measurements; vector control = 100%. (E) Cells were transfected to express HIV-1 Gag (pCMVΔ8.2) and TULA as indicated (6 μg of each plasmid per 100-mm dish) and treated with 20 μg cycloheximide (Sigma) per ml at 40 h post-transfection. Samples of CHX-treated cells were collected and lysed in RIPA at times indicated. Proteins in whole cell lysates were analyzed using Western blotting as indicated. Linear regression was carried out using the Prism software package (GraphPad, San Diego, CA). (F) $CD4^+$HeLa cells were transfected with TULA proteins as indicated and infected with the full-length replication-competent HIV-1 strain RF. Supernatants were harvested at times indicated, and virus production was analyzed using a p24 antigen capture assay. Mean ± SEM of duplicate measurements are shown.

Gag expression. Furthermore, this experiment confirms that the UBA, but not SH3 domain, is essential for the observed effect of TULA.

To corroborate the results obtained using single-round infectivity assays and anti-p24 immunoblotting of viral pellets, we measured the amount of p24 in supernatants of virus-producing 293T cells using a p24 antigen capture assay. Consistent with the previous experiments, a substantial decrease in the level of p24 in supernatants of virus-producing cells was noticed (Fig. 3D). The effect of TULA proteins on the amount of p24 in the supernatant was not as profound as that shown in Figs. 3B and C, probably because a substantial part of p24 in the supernatants is present in the soluble form (probably, due to disintegration of virus-producing cells) and not in virions, thus 'diluting' the observed effect.

Taken together, the results shown in Figs. 3A–D indicate that TULA proteins inhibit production of HIV-1-based viral particles exerting a very modest effect on the intracellular level of HIV-1 Gag. To further characterize the effect of TULA on intracellular HIV-1 Gag, we determined how TULA affects stability of HIV-1 Gag in 293T cells. The results of these experiments indicated that TULA exerts no significant effect on HIV-1 Gag half-life (Fig. 3E), thus confirming our previous findings (Fig. 3C).

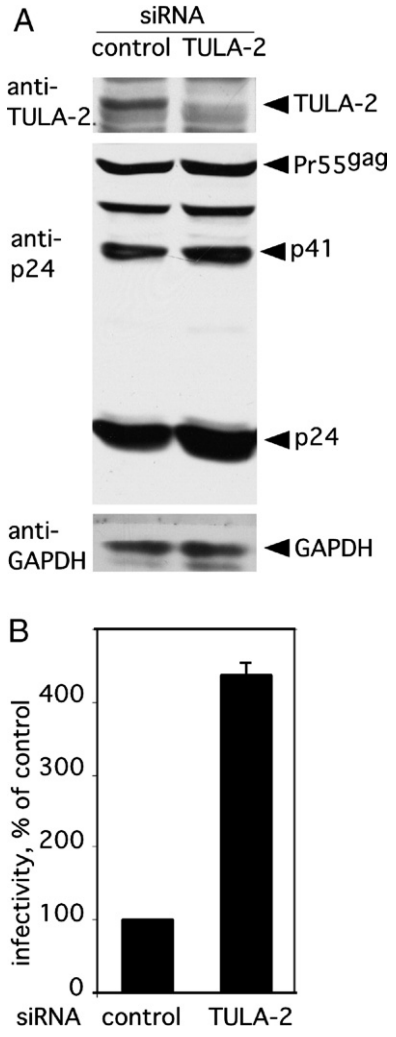

Fig. 4. Effect of TULA-2 depletion on the production of HIV-1. 293T cells were co-transfected with TULA-2-specific or control siRNA twice with a 24-h interval between transfections. The plasmids required for the production of HIV-1-based VSVG-pseudotyped lentivirus (1.5 μg of each pMD.G and pCMVΔ8.2, 3 μg of pCE per well of a 6-well plate) were transfected with the second batch of siRNA. Supernatants of virus-producing cells were harvested. Cells were homogenized, and the obtained homogenates were solubilized in RIPA buffer 40 h after second transfection. (A) RIPA lysates were analyzed using Western blotting. The proteins detected are indicated with arrowheads at the right of the panel. The antibodies used are indicated at the left of the panel. Anti-p24 mAb AG3.0 was used to probe HIV-1 Gag. (B) Cell supernatants were used to transduce Jurkat cells (10 μl aliquots added to 2×10^5 cells in a total volume of 500 μl). Mean ± SD of triplicate measurements are shown; infectivity of control supernatant = 100%. The results of a representative experiment of two independent experiments are shown.

Finally, we evaluated the effect of TULA proteins on the production of replication-competent full-length HIV-1 using overexpression of TULA and TULA-2 in $CD4^+$ HeLa cells. (The cells were selected both to facilitate transfection of TULA-encoding DNA and to support HIV-1 infection and replication.) The results of these experiments indicated that replication of the full-length T-tropic HIV-1 strain RF was decreased approximately 2- to 3-fold in cells transfected to express TULA proteins (Fig. 3F). Therefore, TULA proteins are capable of inhibiting not only production of sub-genomic HIV-1-based virus particles, but also production of virus particles formed by replication-competent full-length HIV-1.

Considering that results obtained using overexpression typically require validation by depletion of an endogenous protein of interest, we depleted TULA-2 in 293T cells using the RNAi approach. (Depletion of TULA was not required, because 293T cells do not express TULA; Feshchenko et al., 2004.) The depletion of TULA-2 was nearly complete (Fig. 4A) and significantly upregulated HIV-1 production in siRNA-treated cells as measured using the single-round infectivity assay (Fig. 4B). The results shown in Fig. 4 indicate that TULA-2 depletion increased infectivity of the produced HIV-1 by a factor of 4.4, while affecting the levels of $Pr55^{gag}$ and p24 to a substantially lower extent (increased 1.2- and 1.7-fold, respectively), thus arguing that the effect of TULA-2 depletion was not mediated simply by modulation of HIV-1 Gag expression. Taken together with the results shown in Fig. 3, these findings are indicative of a negative effect of TULA proteins on HIV-1 biogenesis.

Molecular basis of the effects of TULA on HIV-1 production

Since TULA inhibits production of HIV-1 without exerting a significant effect on HIV-1 Gag expression, we analyzed the pattern of HIV-1 Gag processing in 293T cells transfected to overexpress TULA as being indicative of changes in HIV-1 maturation. These experiments revealed that the presence of TULA typically resulted in the appearance of the p25 band at 72 h post-transfection, which was never seen without TULA overexpression (Fig. 5) or when [ΔUBA]TULA was

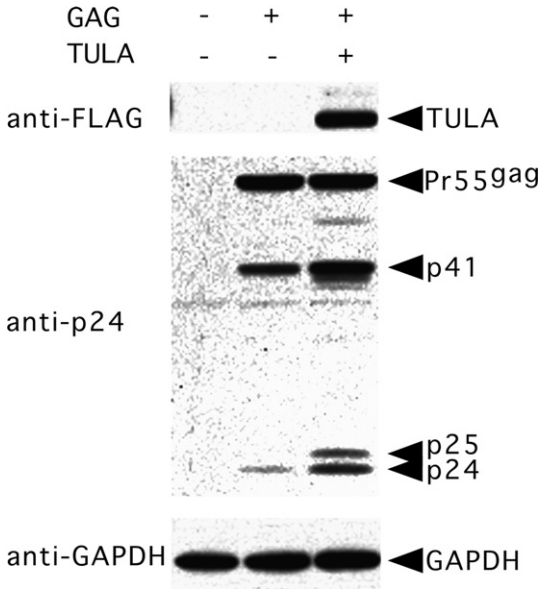

Fig. 5. Effect of TULA on HIV-1 Gag processing. 293T cells were transfected to express HIV-1 Gag and FLAG-tagged TULA in various combinations (8 μg of each plasmid plus 0.5 μg of GFP expression vector per 100-mm Petri dish). The pCMVΔ8.2 plasmid was used to express HIV-1 Gag. Cells were homogenized in hypotonic buffer and solubilized in RIPA buffer 72 h after transfection. Cell lysates were analyzed using Western blotting. The proteins detected and the antibodies used are indicated at the left and at the right of the figure, respectively. Anti-p24 mAb AG3.0 was used to probe HIV-1 Gag. The results of a representative experiment of four independent experiments are shown.

overexpressed (data not shown). This band has previously been shown to be indicative of the impairment of late steps of the HIV-1 life cycle, such as budding and release (Demirov et al., 2002; Gottlinger et al., 1991; Martin-Serrano and Bieniasz, 2003). These findings together with the lack of a substantial effect of TULA proteins on the expression level of HIV-1 Gag (see Figs. 3C, E and 5), argued that TULA acts on HIV-1 production by hampering late steps of the HIV-1 life cycle.

Considering that this effect of TULA may also be manifested in changes of the intracellular localization of HIV-1 Gag, we analyzed intracellular distribution of HIV-1 Gag in the cells overexpressing TULA. First, we examined the effects of TULA on localization of GFP-fused HIV-1 Gag, a fluorescent protein which is fully capable of producing HIV-1 viral particles (Sherer et al., 2003). In the absence of TULA co-expression, HIV-1 Gag-GFP showed punctate localization, primarily to the plasma membrane-proximal area. Upon co-expression of wild-type TULA, plasma membrane-proximal localization of HIV-1 Gag-GFP was diminished, and HIV-1 Gag-GFP was accumulated, to a substantial extent, in the perinuclear area. In contrast, [ΔUBA] TULA did not affect HIV-1 Gag-GFP localization (Fig. 6). Considering that intracellular distribution of HIV-1 Gag may be influenced by other HIV-1 proteins, we next evaluated, using immunofluorescence, the effect of TULA on HIV-1 Gag localization in cells co-transfected with a TULA expression plasmid and the pCMVΔ8.2 plasmid, which encodes all HIV-1 proteins except for Env. In control cells, HIV-1 Gag was localized primarily on the periphery, in the plasma membrane-proximal area, whereas it showed uniform intracellular distribution without any preference for the periphery in TULA-overexpressing cells (Fig. 7A). Therefore, in both systems TULA perturbed intercellular distribution of HIV-1 Gag in a qualitatively similar fashion. The higher extent of the effect of TULA on HIV-1 Gag-GFP may be due to a difference between HIV-1 Gag and HIV-1 Gag-GFP expression levels or to the absence of other HIV-1 proteins in the system shown in Fig. 6.

Since many previous studies implicated $CD63^+$ membrane structures in trafficking of HIV-1 Gag and HIV-1 egress (Deneka et al., 2007; Goff et al., 2003; Jolly and Sattentau, 2007; Jouvenet et al., 2006; Nydegger et al., 2006; Perlman and Resh, 2006; Sherer et al., 2003; von Schwedler et al., 2003; Welsch et al., 2007), we next evaluated co-localization of HIV-1 Gag with CD63, a marker of multivesicular bodies, which is also present on plasma membrane invaginations that serve as sites of productive HIV-1 particle assembly. Partial co-localization of HIV-1 Gag with CD63 was observed in control cells but was diminished upon overexpression of TULA (Fig. 7B) in agreement with the notion that $CD63^+$ compartments are involved in HIV-1 production.

ABCE-1 recruits TULA to HIV-1 Gag

Since TULA binds to ABCE-1, it was possible to speculate that TULA disrupts normal interactions between HIV-1 Gag and ABCE-1, thus inhibiting the HIV-1 capsid assembly by excluding ABCE-1 from assembly intermediates. To evaluate this possibility, we analyzed binding of HIV-1 Gag, ABCE-1 and TULA using their co-immunoprecipitation from cells co-expressing these proteins in various combinations (Fig. 8). (GFP-tagged HIV-1 Gag was used to avoid co-migration of $Pr55^{gag}$ with the heavy chain of anti-HIV-1 Gag antibodies.) First, these experiments confirmed our results indicating that

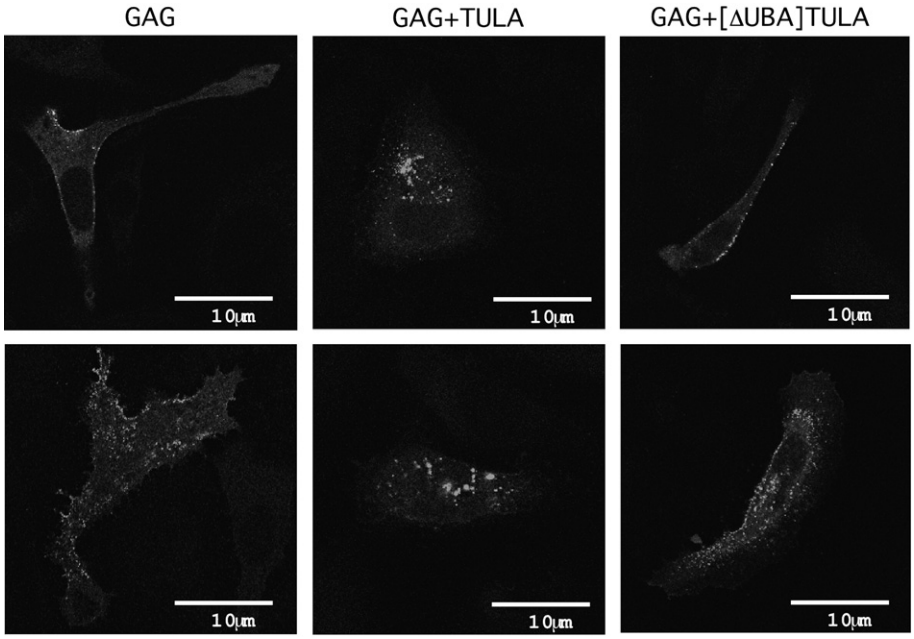

Fig. 6. Effect of TULA on the intracellular localization of HIV-1 Gag-GFP. HeLa cells were transfected to express HIV-1 Gag-GFP and either wild-type TULA or [ΔUBA]TULA as indicated at the top of the figure. In the cells expressing HIV-1 Gag-GFP alone an empty plasmid was transfected instead of a TULA expression plasmid. Cells were fixed and analyzed using a confocal microscope. The scale bar equals 10 μm. Two representative fields are shown for each transfection. The result of a representative experiment of two independent experiments is shown.

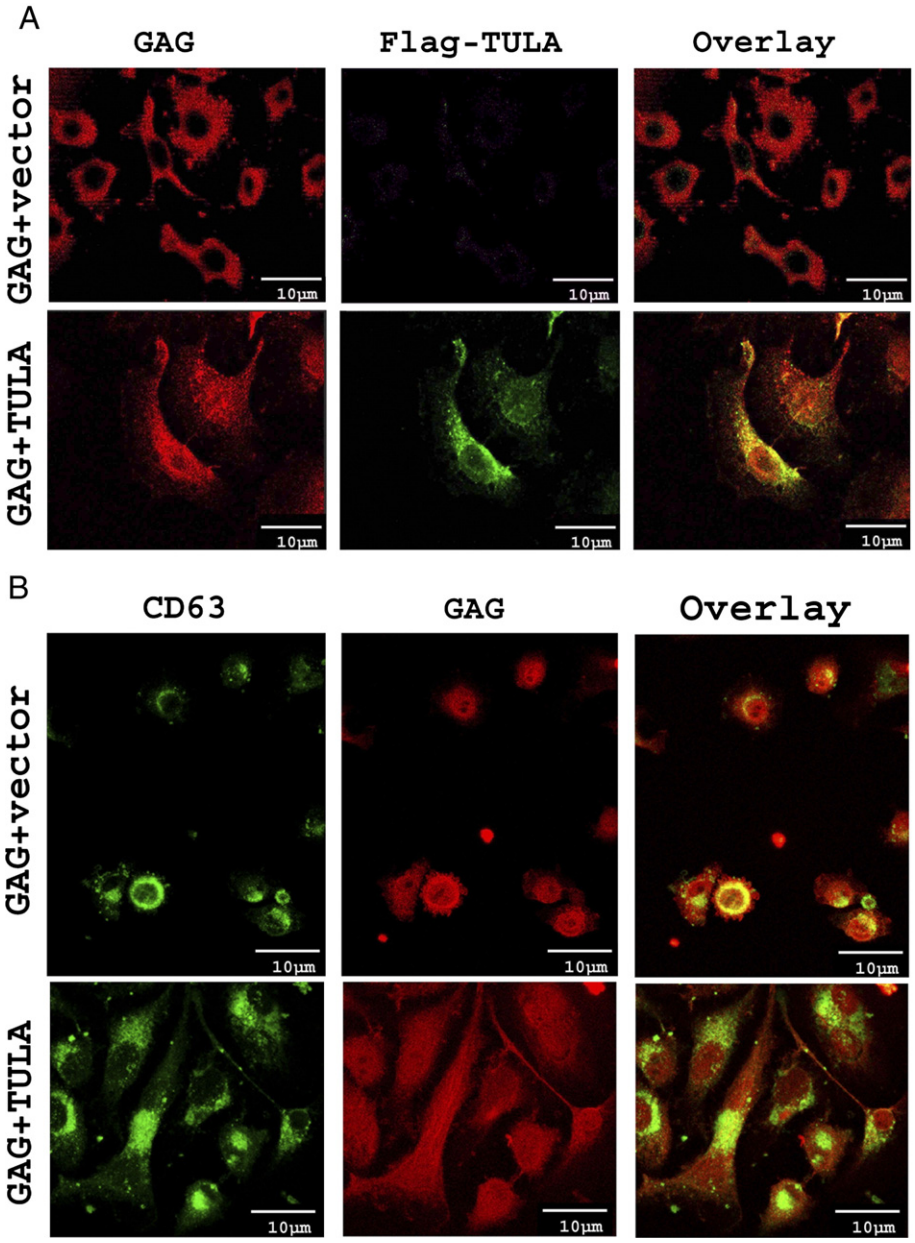

Fig. 7. Effect of TULA on the intracellular localization of HIV-1 Gag. HeLa cells were transfected to express HIV-1 Gag (using pCMVΔ8.2) and wild-type TULA as indicated at the left of each panel (2 μg of each plasmid per 60-mm dish). In the cells expressing HIV-1 Gag alone, an empty plasmid was transfected instead of a TULA expression plasmid. Cells were fixed, stained with the antibodies against HIV-1 Gag and TULA (A) or against HIV-1 Gag and CD63 (B) as indicated at the top of each panel and analyzed using a confocal microscope. The scale bar equals 10 μm. The result of a representative experiment of two independent experiments is shown.

TULA binds to ABCE-1 in a UBA-independent fashion. Second, they confirmed the earlier studies demonstrating binding of ABCE-1 to HIV-1 Gag (Dooher et al., 2007; Zimmerman et al., 2002). Third, they showed that TULA was co-immunoprecipitated with HIV-1 Gag, consistent with the ability of ABCE-1 to bind to both HIV-1 Gag and TULA. However, these experiments failed to provide evidence of the disruption of HIV-1 Gag-ABCE-1 interaction upon overexpression of TULA.

The ability of ABCE-1 to bind to both HIV-1 Gag and TULA (Figs. 2 and 8) and the inability of TULA to disrupt HIV-1 Gag-ABCE-1 binding (Fig. 8) argued that ABCE-1 may act as an adaptor molecule that recruits TULA to HIV-1 Gag. To examine this possibility, we analyzed, using immunoblotting, distribution of HIV-1 Gag, TULA and ABCE-1 in cells overexpressing these proteins in various combinations (Fig. 9). Purity of membrane and cytosolic fractions was verified using VLA-2α and GAPDH, respectively. GFP was used to verify both the efficiency of transfection and the purity of cytosolic fraction. As expected, $Pr55^{gag}$ was localized almost exclusively to the membrane, whereas ABCE-1 and TULA were found both in the cytosol and on the membrane. We analyzed the subcellular distribution of TULA by comparing the ratios of membrane-associated TULA to cytosolic TULA in different

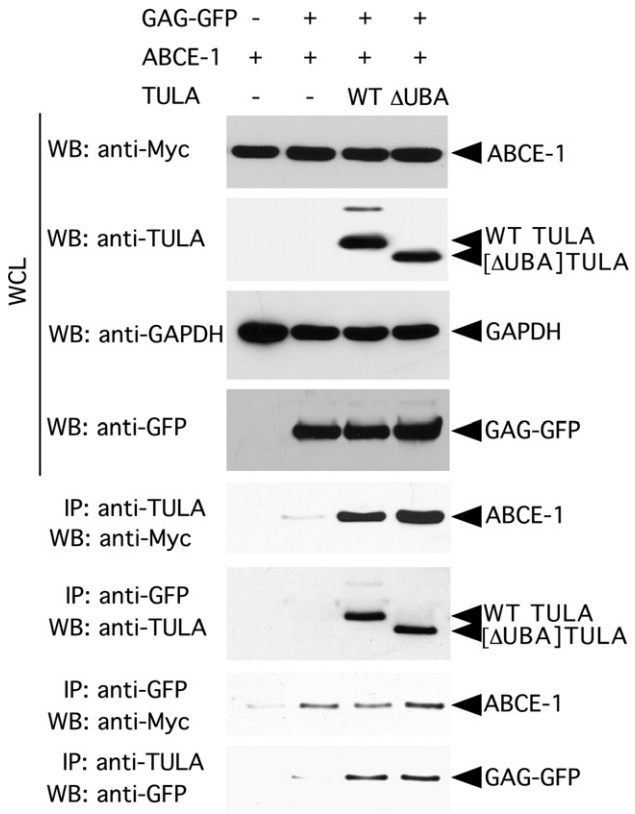

Fig. 8. Interactions of HIV-1 Gag, TULA and ABCE-1. 293T cells were transfected to overexpress GFP-tagged HIV-1 Gag, WT or ΔUBA TULA, and/or Myc-tagged ABCE-1 as indicated at the top of the panel (8 μg of each expression plasmid per 100-mm Petri dish). Cell lysates were obtained 48 h after transfection by homogenizing cells in TNE-L buffer; lysates were filtered through a 0.45-μm filter. Cell lysates and immunoprecipitates were analyzed as described in the legends to Figs. 2 and 3B. The results of a representative experiment of six independent experiments are shown.

samples, since the total level of TULA demonstrated some sample-to-sample variation due to the use of transient expression. Our experiments indicated that the ratio of membrane-associated TULA to cytosolic TULA showed a tendency to increase when TULA was co-expressed with ABCE-1 and, even more so, with both ABCE-1 and HIV-1 Gag (Fig. 9). A moderate increase in the amount of membrane-localized TULA when ABCE-1 was overexpressed alone may possibly be explained by TULA's binding to ABCE-1 associated with the membrane in a Gag-independent fashion. Overall, the results shown in Figs. 8 and 9 suggested that ABCE-1 facilitates the interaction between HIV-1 Gag and TULA, while TULA exerts no substantial effect on the interaction between HIV-1 Gag and ABCE-1. These results are also consistent with partial co-localization of TULA and HIV-1 Gag detected using immunofluorescence (Fig. 7A).

Discussion

The results presented in this report demonstrate that TULA proteins are capable of inhibiting HIV-1 production. Although recent results implicate ABCE-1 in protein translation (Chen et al., 2006; Dong et al., 2004; Estevez et al., 2004; Kispal et al., 2005; Yarunin et al., 2005; Zhao et al., 2004), thus making conceivable that TULA acts on HIV-1 biogenesis by interfering with HIV-1 protein expression, our findings do not support the involvement of this mechanism, since the effect of modulation of the level of TULA proteins on HIV-1 production is much higher than its effect on the expression of HIV-1 Gag (Figs. 3–5). Likewise, TULA neither affects the stability of HIV-1 Gag (Fig. 3E) nor totally blocks processing of HIV-1 Gag, instead slightly increasing the ratio of p24 and p41 to $Pr55^{gag}$ (Figs. 3E and 5). These results, taken together with the ability of TULA to induce the appearance of p25 (Fig. 5), a change in HIV-1 Gag processing pattern that is consistent with a block in HIV-1 budding and release (Demirov et al., 2002; Gottlinger et al., 1991; Martin-Serrano and Bieniasz, 2003), indicate that TULA proteins inhibit late steps of the HIV-1 life cycle. Importantly, our results indicate that TULA proteins inhibit production of viral particles by both sub-genomic and full-length replication-competent HIV-1 (Fig. 3), arguing that this effect is biologically relevant. It should be noted that overexpressed TULA proteins were incapable of inhibiting HIV-1 infection at MOI 10-fold higher than that used in the experiment presented in Fig. 3F (data not shown). This may be due simply to the saturation of TULA or to an active counter-effect of HIV-1.

The findings that TULA and TULA-2, when overexpressed, induce similar effects on HIV-1 production as judged using multiple experimental approaches (Figs. 3A, B, D, F) and that the effect of TULA-2 depletion, i.e. an increase in HIV-1

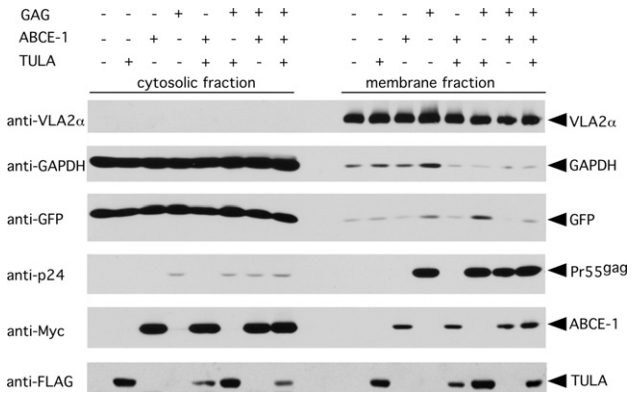

Fig. 9. Effect of HIV-1 Gag and ABCE-1 on the subcellular distribution of TULA. 293T cells were transfected to overexpress HIV-1 Gag, FLAG-tagged TULA and/or Myc-tagged ABCE-1 as indicated at the top of the panel (8 μg of each expression plasmid per 100-mm Petri dish). Empty vector was added where required to keep the total amount of DNA constant. GFP expression plasmid was added to each sample to monitor the efficiency of transfection (0.5 μg per 100-mm Petri dish). Cells were homogenized 48 h after transfection. Membrane and cytosolic fractions were obtained using ultracentrifugation of pre-cleared homogenate and analyzed using Western blotting. The proteins detected and the antibodies used for detection are indicated at the right and at the left of the corresponding panels, respectively. Anti-p24 mAb AG3.0 was used to probe HIV-1 Gag. The level of membrane and cytosolic TULA in the relevant samples has been quantified. The results of a representative experiment of four independent experiments are shown. Assigning the value of 1.0 to the ratio of membrane-associated TULA to cytosolic TULA in cells transfected to express TULA alone, these ratios were 1.41±0.37, 0.99±0.21 and 1.84±0.56 (mean±SEM) for TULA/ABCE-1, TULA/GAG and TULA/ABCE-1/GAG cells based on all four experiments.

production as assessed using the single-cycle infectivity assay, is consistent with the effect of overexpressed proteins (Fig. 4) argue that the observed inhibitory effect is common for both family members. This finding is in agreement with the critical role of the UBA domain in the observed effects (Figs. 3A–C), since the structure and ubiquitin-binding ability are similar for the UBA domains of TULA and TULA-2 (Carpino et al., 2004; Feshchenko et al., 2004; Hoeller et al., 2006; Kowanetz et al., 2004). The essential role of the UBA domain in the TULA-dependent inhibition of HIV-1 production argues that TULA is likely to act by disrupting the ubiquitylation-mediated events critical for the late steps of the HIV-1 life cycle.

Several lines of evidence, including the impairment of HIV-1 release from HIV-1 Gag-producing cells by decreasing the cellular pool of ubiquitin or by mutating multiple HIV-1 Gag ubiquitin-acceptor sites, suggest a functional contribution of ubiquitylation to retroviral budding, in general, and that of HIV-1, in particular (Gottwein et al., 2006; Gottwein and Krausslich, 2005; Jager et al., 2007; Patnaik et al., 2000; Schubert et al., 2000; Strack et al., 2000, 2002). The molecular basis of this effect is not fully understood but may be related to the recruitment, for example, to ubiquitylated HIV-1 Gag, of cellular factors that are essential for both virus budding and the formation of intraluminal vesicles of the multivesicular bodies, such as the ESCRT-I complex (Bieniasz, 2006; Morita and Sundquist, 2004). HIV-1 Gag appears to interact with the ESCRT-I complex through Tsg101, a protein binding to both the PTAP motif and ubiquitin (Garrus et al., 2001; Goff et al., 2003; Stuchell et al., 2004; von Schwedler et al., 2003). Although binding of Tsg101 to HIV-1 Gag may involve several different interactions, it has been shown that the loss of ubiquitin binding by Tsg101 prevents its interaction with HIV-1 Gag and greatly reduces production of virus-like particles (Goff et al., 2003). Therefore, TULA may interfere with the interactions between HIV-1 Gag and Tsg101 by competing with Tsg101 for a crucial ubiquitin. It should be noted that this ubiquitin may be attached to proteins other than HIV-1 Gag. For example, the novel E3 ubiquitin-protein ligase POSH has been shown to be essential for HIV-1 production, while apparently incapable of ubiquitylating HIV-1 Gag (Alroy et al., 2005). Finally, we cannot rule out that TULA exerts its effect on the late steps of HIV-1 life cycle by regulating the level of ubiquitylation of a certain crucial protein, which may or may not be HIV-1 Gag.

The location of sites of productive HIV-1 particle assembly has been a matter of controversy. Multiple studies suggested previously that assembly in late endosomes is a general feature of retroviruses, including HIV-1 (Blot et al., 2004; Goff et al., 2003; Nydegger et al., 2003; Pelchen-Matthews et al., 2003; Perlman and Resh, 2006; Sherer et al., 2003). However, several recent studies demonstrated that the primary site of productive HIV-1 particle assembly in various cell types is plasma membrane and that the presence of HIV-1 particles in late endosomes is due to their internalization from the plasma membrane (Deneka et al., 2007; Jolly and Sattentau, 2007; Jouvenet et al., 2006; Nydegger et al., 2006; Welsch et al., 2007). Notably, these studies indicate that the plasma membrane structures in which HIV-1 particles are assembled and budded are positive for the endosomal marker CD63, thus rendering $CD63^+$ compartments critical for HIV-1 biogenesis according to both models of HIV-1 Gag trafficking. Therefore, we characterized the effect of TULA on the critical features of intracellular localization of HIV-1 Gag, i.e. its plasma membrane/intracellular distribution and its co-localization with CD63. These experiments revealed that TULA diminishes both localization of HIV-1 Gag to the plasma membrane-proximal area (Figs. 6 and 7A) and its co-localization with CD63 (Fig. 7B), thus being consistent with the findings demonstrating inhibition of the late steps of the HIV-1 life cycle by TULA proteins. The identity of compartments to which HIV-1 Gag is localized in the presence of TULA remains to be determined.

Our results provide no direct support to the hypothesis that TULA inhibits HIV-1 biogenesis by disrupting the interaction of ABCE-1 with HIV-1 assembly intermediates, while suggesting the role of ABCE-1 as a recruiter of TULA to HIV-1 Gag (Figs. 8 and 9). The notion that TULA is recruited to HIV-1 Gag is consistent with the partial co-localization of these proteins (Fig. 7A). The translocation of TULA to the sites of HIV-1 assembly, although partial, is likely to be important for providing specificity to the observed effect of TULA, since TULA proteins appear to exert multiple UBA-mediated effects (Feshchenko et al., 2004; Hoeller et al., 2006; Kowanetz et al., 2004). It should also be noted that the obtained results cannot entirely rule out the possibility that TULA inhibits HIV-1 biogenesis by adversely affecting HIV-1 Gag-ABCE-1 interactions, because it is conceivable that TULA interferes with these interactions and hence with the biological effect of ABCE-1 without significantly decreasing co-immunoprecipitation of HIV-1 Gag and ABCE-1. Clearly, the molecular basis of the functional link between TULA and ABCE-1 in the observed phenomena remains to be understood.

Notably, TULA is not the sole protein capable of exerting a negative effect on HIV-1 biogenesis; multiple proteins have been shown to mediate cellular defense against HIV-1 infection. Thus, APOBEC-family proteins act by hypermutating nascent reverse transcripts of HIV-1 (Gaddis et al., 2003; Harris et al., 2003; Lecossier et al., 2003; Mangeat et al., 2003; Mariani et al., 2003; Zhang et al., 2003) and by preventing the accumulation of reverse transcripts (Bishop et al., 2006; Newman et al., 2005). TRIM5α, another intracellular anti-HIV factor, appears to act by promoting rapid, premature disassembly of retroviral capsids (Perez-Caballero et al., 2005; Stremlau et al., 2004, 2006, 2005; Yap et al., 2004). OTK18 has been shown to act through suppression of HIV-1 transcription (Carlson et al., 2004). TULA proteins appear to inhibit the late steps of the HIV-1 life cycle through a mechanism dissimilar to any of those known. It remains to be determined whether the molecular basis of the observed effect of TULA proteins is unique or other proteins exist that also exert a negative effect on the HIV-1 life cycle by interfering with its critical ubiquitylation-dependent events. Finally, our results suggest that the expression of HIV-1 protein is capable of reducing the endogenous level of TULA-2 (data not shown), but whether or not this phenomenon represents a counter-effect of HIV-1 on the cellular defense mediated by TULA proteins has to be elucidated further.

Materials and methods

DNA constructs and siRNA

Expression constructs for V5-tagged wild-type and mutant forms of TULA based on pAlterMAX described previously (Feshchenko et al., 2004) were used as a source of the corresponding cDNA. To obtain FLAG-tagged TULA, cDNA encoding wild-type TULA short (Feshchenko et al., 2004) was subcloned into the pFLAG 5a vector (Sigma, St. Louis, MO) between *Eco*RV and *Kpn*I restriction sites using the Advantage-Hf2 polymerase (Clontech, Mountain View, CA). Forward and reverse primers annealed with 15–18 nucleotides of the TULA sequence and included the corresponding restriction site. To introduce mutations, two oligonucleotides complementary to the opposite strands of double-stranded DNA containing the sequence to be mutated were designed to contain 15–18 nucleotides on either side of the mutation site. The mutagenesis reactions were performed using the QuikChange Site-Directed Mutagenesis Kit according to the manufacturer's recommendations (Stratagene, La Jolla, CA). To obtain Myc-tagged ABCE-1, cDNA encoding human ABCE-1, which was previously subcloned into the pcDNA3 vector (Martinand et al., 1999), was amplified using the Expand High Fidelity PCR System (Roche Applied Science, Indianapolis, IN). The forward primer included an *Eco*RI restriction site and the coding sequence for the Myc tag. The obtained fragment was ligated into the pAlterMAX vector between its *Eco*RI and *Sma*I restriction sites. All resulting constructs were verified by sequencing. To assess lentiviral production, pCE, an HIV-based transfer plasmid encoding for green fluorescent protein (GFP) (Hasham and Tsygankov, 2004); pCMVΔ8.2, a packaging plasmid encoding for all HIV-1 proteins except Env; and pMD.G, an envelope plasmid encoding for the vesicular stomatitis virus glycoprotein (VSV-G) were used (Naldini et al., 1996). The pGag-GFP plasmid was constructed by PCR fusion of Rev-independent full-length *gag* gene to GFP in pEGFP-N1 (Clontech) (Hermida-Matsumoto and Resh, 2000). To deplete TULA-2/Sts-1, ON-TARGETplus SMARTpool siRNA (L-008533-00; Dharmacon, Lafayette, CO) or an equimolar mix of three individual siRNA (ID#121987, 121986, 34950; Ambion, Austin, TX) was used. To deplete c-Cbl, an equimolar mix of two individual siRNA (ID#121527 and 121528: Ambion) was used. Silencer Negative Control #1 siRNA (Ambion) was used as negative control.

Cells and transfection

HEK293T and MAGI cells (both obtained from ATCC) were cultured in DMEM supplemented with 2 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin and 10% fetal bovine serum (FBS). (MAGI are HeLa cells stably expressing CD4 to facilitate HIV-1 infection and containing a genome-integrated *lac*Z gene under control of the HIV-1 LTR promoter; Kimpton and Emerman, 1992.) T-cell lines (Jurkat, MOLT4 and SupT1—all obtained from ATCC) were cultured in RPMI1640, which was supplemented as described above for DMEM. 293T cells were transfected with purified plasmid DNA using Lipofectamine-2000 (Invitrogen/Life Technologies, Carlsbad, CA) according to the manufacturer's recommendations and harvested 36 to 72 h after transfection. When required, a GFP-encoding expression plasmid (pEGFP-C2, Clontech) was co-transfected with other expression plasmids to assess the efficiency of transfection. Empty vector DNA was added to the transfection mix, where necessary, to maintain total DNA amount constant. MAGI cells were transfected using DMRIE-C (Invitrogen/Life Technologies) according to the manufacturer's recommendations. Forty-eight hours after transfection, cells were harvested and re-seeded in multiple wells and infected with the full-length replication-competent T-tropic (X4) HIV-1 strain RF at MOI ~0.05. The numbers of cells in transfected cultures were determined prior to infection. Supernatants of infected cells were harvested 48 and 96 h post-infection and frozen. The RF strain was obtained from the AIDS Research and Reference Reagent Program (NIAID, Rockville, MD), propagated in MOLT4 cells, purified from culture supernatants using ultracentrifugation and titrated using a syncytia assay with SupT1 cells as described previously (Raymond et al., 2006, 2004). All handling of HIV-1 was carried out in a BSL2+ facility using standard safety procedures.

Cell lysis and subcellular fractionation

Several lysis protocols were used to minimize non-specific co-immunoprecipitation in each type of experiment. TNE buffer (50 mM Tris, 150 mM sodium chloride and 2 mM EDTA; pH 7.6) contained 1% NP-40 (Feshchenko et al., 1998). TNE-L buffer contained 10 mM Tris (pH 7.4), 100 mM sodium chloride, 50 mM potassium acetate, 10 mM EDTA and 1% NP-40 (Zimmerman et al., 2002). RIPA buffer was made by adding SDS and sodium deoxycholate to TNE at the final concentrations of 0.1 and 0.5%, respectively. In the experiments in which subcellular localization of proteins was studied, cells were homogenized prior to solubilization by being passed 10–20 times through a 19- to 25-gauge needle in hypotonic buffer (10 mM Tris, 0.5 mM $MgCl_2$; pH 7.5). Tonicity was restored by adding 0.25 ml of high-salt buffer (same as hypotonic, but containing 0.6 M sodium chloride) to 1 ml of hypotonic homogenate, the resultant mix was centrifuged at 750×*g* for 5 min to remove unbroken cells and nuclei, and 0.5 mM EDTA was added to post-nuclear supernatant. Finally, concentrated TNE/β-octyl-D-glucoside (TNE/ODG) buffer was added to post-nuclear supernatant to final concentrations of 1xTNE and 2% ODG. In all cases except for studying subcellular fractionation, cells or cell homogenates were solubilized for 30 min, and then lysates were cleared by centrifugation (12,000×*g*, 5 min). All buffers used for lysis/homogenization were supplemented with inhibitors (10 μg/ml of each aprotinin, leupeptin and pepstatin, 0.5 mM PMSF, 10 mM sodium fluoride, 1 mM pervanadate), and all lysis procedures were carried out at +4 °C. To study subcellular distribution of proteins, cells were transfected and homogenized as described above. Post-nuclear supernatant was fractionated further using ultracentrifugation at 100,000×*g* for 50 min at +4 °C. The obtained supernatant and pellet represented cytosolic and membrane fractions, respectively. The

pellet was washed with phosphate-buffered saline (PBS) and solubilized in RIPA buffer for 30 min on ice.

Lentivirus production

To assess the effect of TULA and other proteins on HIV-1 production, 293T cells were grown to be ~30% confluent on the day of transfection. Purified plasmids required for lentivirus production were transfected into 293T cells using calcium phosphate, and virus-containing supernatants were harvested and filtered through a 0.45-μm filter 3 days after transfection (Hasham and Tsygankov, 2004). Virus production was assessed using a single-round infectivity assay and biochemical analysis of purified virions. The single-round infectivity assay was based on flow cytometry-based enumeration of target cells infected with a replication-incompetent virus encoding for GFP (Alroy et al., 2005): filtered viral supernatants were added to Jurkat cells in 24-well plates (2×10^5 cells/0.5 ml of medium per sample) in the presence of 8 μg/ml polybrene, and cells were spin-inoculated at 800×*g* for 90 min at 32 °C. Jurkat cells were analyzed using flow cytometry 2 days after spin-inoculation. The percentage of infected (GFP^+) cells showed the linear dependence on the volume of supernatant in the range from 2 to 100 μl (corresponding to the multiplicity of infection from ~0.01 to ~0.5). To assess virus production using Western blotting, supernatant was centrifuged through a 20% sucrose cushion at 50,000×*g* for 90 min at +4 °C. To study the effect of protein depletion on virus production, 293T cells were grown to be 20–30% confluent and transfected with 150 nM total siRNA using X-treme reagent (Roche Applied Sciences) at an RNA:reagent ratio of 1:5 (w/v) in 2.5 ml of Opti-MEM per well of a 6-well plate. Cells were re-transfected with 150 nM siRNA and the plasmids required for virus production using calcium phosphate 24 h after the initial siRNA transfection. Supernatant was collected 40 h after the second transfection and analyzed as described above in this section. Virus-producing cells were lysed for biochemical analysis in all experiments when supernatants were harvested.

Immunofluorescence microscopy

HeLa cells were grown to be ~80% confluent and transfected with the plasmid encoding for HIV-1 Gag-GFP (for direct analysis of fluorescence) or the pCMVΔ8.2 plasmid (for immunofluorescence staining) and a pAlterMAX plasmid encoding for either wild-type or ΔUBA TULA (1 μg of each plasmid per well of a 6-well plate) using DMRIE-C as per the manufacturer's recommendations. One day later, cells were harvested and seeded on glass coverslips pre-coated with fibronectin (10 μg/ml, overnight, +4 °C) at a confluency of ~50%. Six hours later, the cells were washed, fixed with 3.7% formaldehyde in PBS for 20 min at +4 °C with shaking. Cells expressing HIV-1 Gag-GFP were washed in PBS containing 3% FBS followed by equilibration buffer (Molecular Probes, Eugene, OR). Cells expressing HIV-1 Gag were further incubated in 50 mM glycine-containing PBS to quench free aldehyde groups and permeabilized in PBS containing 2% BSA and 0.1% Triton X-100. HIV-1 Gag, FLAG-TULA and CD63 were stained with the corresponding antibodies at room temperature for 1 h. Polyclonal rabbit anti-HIV-1 p24 antibody H6003-33E from US Biologicals (Swampscott, MA), anti-FLAG M2 mouse monoclonal antibody (mAb) from Sigma and anti-CD63 H5C6 mouse mAb from BD Pharmingen (San Diego, CA) were diluted in permeabilizing buffer at final concentrations of 20, 5 and 2 μg/ml, respectively. Coverslips were extensively washed with PBS and stained with the corresponding secondary antibodies at room temperature for 1 h. Goat anti-mouse IgG-FITC (Southern Biotech, Birmingham, AL) and bovine anti-rabbit IgG-rhodamine (Santa Cruz Biotechnology, San Cruz, CA) were used at the dilutions of 1:100 and 1:400, respectively. The coverslips were mounted onto a slide with anti-fade mounting solution (Molecular Probes). Cell images were obtained using confocal microscopy (Leica DM IRE2 microscope with a 100× objective).

Adenovirus

Adenoviruses expressing wild-type and mutant FLAG-tagged TULA were constructed using the AdEasy Vector System (Q-Biogene, Carlsbad, CA) essentially as described previously (Fikaris et al., 2006). cDNA encoding FLAG-tagged TULA was amplified using the Expand High Fidelity PCR System (Roche Applied Science) with the corresponding cDNA TULA (wild-type, W279L or ΔUBA) subcloned into pAlterMAX as templates. The forward primer annealed to the 18 nucleotides corresponding to the N-terminal end of TULA and included a *Bam*HI restriction site and the coding sequence for the FLAG tag. The reverse primer annealed to the 17 nucleotides corresponding to the C-terminal end of TULA and included a stop codon and a *Spe*I restriction site. The introduced sites were used to ligate the FLAG-TULA cDNA into the pShuttle-CMV vector by generating sticky ends compatible with those formed by digesting pShuttle-CMV with *Bgl*II and *Xba*I. The resulting constructs were verified by sequencing and used for recombination with adenoviral DNA in BJ5183 competent *E. coli* cells. QBI-293A cells were transfected with recombinants and analyzed for adenovirus production using plaque assay. The selected clones were propagated as per the manufacturer's recommendations, and the titers of final preparations were determined using plaque assay. LacZ-expressing adenovirus was used as negative control.

Identification of TULA-associated proteins

293T cells were transfected with the FLAG-TULA expression plasmid, harvested, washed with PBS and lysed in CelLytic buffer (Sigma) for 15 min at room temperature. Cell debris was removed by centrifugation, and 1–3 mg of total protein from FLAG-TULA-expressing or vector-transfected cells was incubated with 20 μl anti-FLAG M2 affinity gel (Sigma) at 4 °C for 4 h. The beads were washed three times with CelLytic buffer, and anti-FLAG-bound proteins were eluted from the beads with 0.1 M glycine (pH 3). Proteins eluted from the anti-FLAG beads were separated on a one-dimensional Bis–Tris minigel and stained in Simply Blue Coomassie (Invitrogen). After in situ

trypsin digestion (Joyal et al., 1997), proteins were analyzed by data-dependent LC-MS/MS on an Agilent LC-MSD. Uninterpreted mass spectra were searched against a human non-redundant protein database using Mascot (Matrix Science) (Perkins et al., 1999). Errors used were 2.0 kDa on MS data and 0.8 kDa on MS/MS data.

Immunoprecipitation and immunoblotting

Total protein (1–3 mg) from whole cell lysate was incubated with an antibody indicated (1–3 μg/sample) in a total volume of 1 ml and immunoprecipitated with 50 μl of Protein A Sepharose (Amersham Biosciences, Piscataway, NJ) or 20 μl of Protein G Agarose (Upstate Biotechnology, Lake Placid, NY) slurry. Protein A Sepharose was used to precipitate rabbit antibodies, whereas all other antibodies were precipitated using Protein G Agarose. Proteins were separated using SDS–PAGE, transferred to nitrocellulose and probed with the corresponding antibody at an appropriate dilution as indicated. The appropriate peroxidase-conjugated secondary antibody was added, and proteins were visualized using chemiluminescence. Anti-Myc 4A6 mouse mAb was purchased from Upstate Biotechnology. Anti-ABCE1 NB400-116 rabbit polyclonal antibody was purchased from Novus Biologicals (Littleton, CO). Anti-HIV-1 Gag antibodies recognizing both p24 and unprocessed $Pr55^{gag}$ (AG3.0 mouse mAb and sheep polyclonal antiserum, catalogue #4121 and catalogue #287, respectively) were obtained through the NIH AIDS Research and Reference Reagent Program (Germantown, MD). Anti-p24 goat polyclonal antibody recognizing only the processed p24 form was purchased from ViroStat (Portland, ME). Anti-p17 chicken polyclonal antibody recognizing only p17 was purchased from Genway (San Diego, CA). Anti-VLA2α mouse mAb was purchased from BD Biosciences. Anti-GFP B-2 mouse mAb was purchased from Santa Cruz Biotechnology. Anti-GAPDH 6C5 mouse mAb was purchased from Research Diagnostics (Flanders, NJ). Anti-TULA and anti-TULA-2 rabbit polyclonal antibodies were custom-produced by Proteintech Group (Chicago, IL) against the peptides containing amino acids 4–23 of TULA and 15–34 of TULA-2/Sts-1, respectively, conjugated to KLH and affinity purified using the corresponding antigen peptide.

Antigen capture enzyme-linked immunoassay (ELISA)

HIV-1 p24 protein was measured in cell supernatants using an HIV-1 p24 Antigen ELISA kit from ZeptoMetrix (Buffalo, NY) or SAIC (Frederick, MD). Briefly, p24 was captured by a plate-immobilized anti-p24 antibody and detected using a biotinylated anti-p24 followed by streptavidin-peroxidase (ZeptoMetrix) or rabbit anti-p24 followed by anti-rabbit goat IgG-peroxidase (SAIC). The amount of p24 in specimens was quantified using its comparison to a standard curve.

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